Chapter 4

Isolation of Breast Cancer Stem Cells by Single-Cell Sorting

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1. Introduction

Breast cancer is the most common cancer in women, with more than 1,000,000 new cases and more than 410,000 deaths each year [38]; [39]. At present, breast cancer is mainly treated by surgical therapy as well as cytotoxic, hormonal and immunotherapeutic agents. These methods achieve response rates ranging from 60 to 80% for primary breast cancers and about 50% of metastases [22]; [24]. However, up to 20 to 70% of patients relapse within 5 years [10].

The reason for recurrence is the existence of cancer stem cells in malignant tumors such brain, prostate, pancreatic, liver, colon, head and neck, lung and skin tumors [3]; [7]; [14]; [15]; [21]; [32]; [49]; [51]. Breast cancer stem cells (BCSCs) were first detected by Al-Hajj et al. (2003) that showed cells expressing CD44 protein and weakly or not expressing CD24 protein could establish new tumors in xeno-grafted mice. Using these markers, researchers isolated BCSCs from primary [41]; [47] and established breast cancer cell lines [16]. Another technique used is cell culture in serum-free medium to form mammospheres. Mammospheres exhibit many stem cell-like properties such as differentiation into all three mammary epithelial lineages [11]; [12]. These BCSCs have been demonstrated to cause treatment resistance and relapse. Thus, BCSC-targeting therapy is considered a promising therapy for treating breast cancer.

Recently, BCSC-targeting therapies have been researched by various groups worldwide. Strategies include targeting the self-renewal of BCSCs [30]; [31], indirectly targeting the microenvironment [29]; [50]; [31] and directly killing BCSCs by chemical agents that induce differentiation [25]; [19]; [42]; [43], immunotherapy [4]; [5]; [40] and oncolytic viruses [26]; [34]. In all strategies, isolation of BCSCs is an important step to recover starting materials for all subsequent steps. Thus, isolation of BCSCs is a pivotal step for successful outcomes. Almost all

studies have focused more on treatment strategies than isolation of BCSCs. Indeed, to date, there are only three methods used to identify and isolate BCSCs, namely fluorescence-activated cell sorting (FACS) based on BCSC markers such as CD44, CD24 and CD133 [2]; [52]; [46]; [41], identification of the side population (SP) that effluxes Hoechst 33342 [13]; [8]; [28] and mammosphere formation [44]; [54]. All these methods possess some limitations.

The first limitation is the resulting heterogenous population of BCSCs. Using these techniques, the BCSC population contains phenotypes with differences in CD44 and CD24 expression levels. These differences reflect variations in some cellular behaviors. BCSCs isolated by SP sorting or mammosphere culture may contain a small population that do not exhibit the CD44+CD24- phenotype. Thus, in this study, we attempted to establish a new method to isolate a homogenous population from malignant breast tumors.

Our study is based on the cell cloning technique that is applied to select hybridomas for monoclonal antibody production. Using a cell sorter with the index sorting function, we aim establish a new protocol that can isolate and establish BCSC clones at a high efficiency.

2. Materials and methods

2.1. Primary culture

Primary culture of breast cancer cells from malignant breast tumors was carried out as described elsewhere [41]; [42]. Tumor biopsies were obtained from consenting patients at the Oncology Hospital in Ho Chi Minh city, Vietnam, and then transferred to the laboratory on ice. All samples were kept in phosphate-buffered saline (PBS) containing 1× antibiotics and an antimycotic (Sigma-Aldrich, St Louis, MO). Tumors were homogenized into small fragments (approximately 1–2 mm3) using scissors. These samples were seeded in 35-mm culture dishes (Nunc, Roskilde, Denmark) in M171 medium (Invitrogen, Carlsbad, CA) containing mammary epithelial growth supplement (MEGS) (Invitrogen, Carlsbad, CA), and incubated at 37°C with 5% CO2. Five patients participated in this study.

2.2. Single-cell sorting

Primary cells were detached by 0.25% trypsin/EDTA. The cell suspension was washed twice with PBS to eliminate trypsin. The cell pellet was resuspended in sorting buffer (PBS containing 0.2 mM EDTA and 1 mg/mL bovine serum albumin (BSA) at $1x10^6$ cells/ml. Single-cell sorting was performed on a BD FACSJazz (BD Bioscience, Franklin Lakes, NJ) using the index sorting function. One cell was sorted into one well of a 96-well plate that contained M171 medium with MEGS. One sample was sorted into 2880 wells in 30 plates. After sorting, all wells were checked for a single cell/well under an inverted microscope.

2.3. Single cell-based culture and selection of mesenchymal-like cells

After single-cell sorting, cells were cultured in M171 medium containing MEGS and incubated at 37°C with 5% CO2. Half medium volumes were exchanged every 3 days for 30 days. Then,

only wells that contained cell colonies were used to select mesenchymal-like cell clones by replacing M171 medium with DMEM/F12 supplemented 10% fetal bovine serum (FBS) for 2 days. In this medium, all epithelial-like cells did not survive. Surviving mesenchymal-like cell clones were continuously subcultured for three to five passages. Cell clones that rapidly underwent an epithelial-mesenchymal transition (EMT) were considered as BCSC candidates. These cells were used to analyze some characteristics of BCSCs in subsequent experiments.

2.4. $CD44^{+}CD24^{-/dim}$-based cell sorting

BCSCs were isolated from primary cultures based on CD44+CD24- expression by FACS as described elsewhere [42]. Briefly, 1 ml cell suspensions in PBS ($1x10^7$ cells) were double stained with 20 μl anti-CD44-FITC and 20 μl anti-CD24-PE. Samples were incubated in the dark at room temperature for 45 min. The CD44+CD24-/dim cell population was identified by the software controlling the BD FACSJazz. Cells were sorted into 2-ml tubes containing 1 ml culture medium (DMEM/F12 supplemented with 10% FBS and a 1× antibiotic-mycotic (Sigma-Aldrich, St Louis, MO).

2.5. Rhodamine 123 efflux and SP analysis

Cells were stained with 0.1 μg/mL rhodamine (Sigma-Aldrich) at 37°C for 30 min, and then washed twice with 2% FBS in PBS. Two filters (FL1 and FL3) were used to detect rhodamine 123. Cells incubated with 50 μM verapamil and 0.1 μg/ml rhodamine 123 for 30 min were used as a positive control.

2.6. Immunophenotyping

Cell markers were analyzed following a previously published protocol [43]. Briefly, cells were washed twice in PBS containing 1% BSA (Sigma-Aldrich). Cells were stained for 30 min at 4°C with anti-CD13-FITC, anti-CD90-PE and anti-CD133-PerCP monoclonal antibodies (BD Biosciences). Stained cells were analyzed by a BD FACSCalibur (BD Biosciences) flow cytometer. Isotype controls were used in all analyses.

2.7. Mammosphere culture

Cells were detached with 0.25% trypsin/EDTA and resuspended in serum-free DMEM/F12 (1:1; GeneWorld, Ho Chi Minh, VN) containing 15 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor (EGF), 2 mM/l L-glutamine, 4 U/l insulin growth factor (Sigma-Aldrich) and B27 supplement (1:50; Invitrogen). Cells were cultured at 37°C with 5% CO2.

2.8. Cell cycle analysis

Cells were washed twice in PBS and fixed in cold 70% ethanol for at least 3 h at 4°C. Then, cells were washed twice in PBS and stained with 1 ml PI (20 μg/ml). Fifty microliters of RNase A (10 μg/ml) was added to samples, followed by incubation for 3 h at 4°C. Stained cells were analyzed by flow cytometry using CellQuest Pro software (BD Biosciences).

2.9. Doxorubicin resistance analysis

BCSCs were cultured to 10^4 cells/well in a 24-well plate (Nunc, Roskilde, Denmark), in DMEMF12/10% FBS. After 24 h culture to confluence, cells were treated with 0, 1 and 3 μg/ml doxorubicin (Sigma-Aldrich). Doxorubicin resistance was analyzed by apoptosis using annexin-V-FITC and PI on a FACSCalibur.

2.10. *In vivo* tumorigenesis

Non-obese diabetic (NOD)/SCID mice (5–6-weeks-old) (NOD.CB17-Prkdcscid/J; Charles River Laboratories) were used in this study. All mice were housed in clean cages and maintained according to institutional guidelines on animal welfare. Mice were subcutaneously injected with $1x10^6$, 10^5, 10^4 and 10^3 cells (n=3, each dose). Mice were followed up for 1 month to detect tumors.

2.11. Statistical analysis

All experiments were performed in triplicate. A value of $P \leq 0.05$ was considered significant. Data were analyzed using Statgraphics v 7.0 software (Statgraphics Graphics System, Warrenton, VA).

3. Results

3.1. Primary culture

The study was carried out to primary culture five tumors from five patients. There were 3/5 samples that outgrew cells (Fig. 1A). These cells from the three samples were propagated until 80% confluence. In almost all samples, epithelial-like cells appeared before mesenchymal-like cells, which spread out from tumor fragments from day 5. Mesenchymal-like cells usually appeared at day 20. Then, cells proliferated rapidly and formed colonies. At this time, two cell shapes were mostly observed in the primary culture (Fig. 1B). These were epithelial-like cells with a bean shape and large nucleus, and mesenchymal-like cells with a small nucleus and elongated shape. Cells were subcultured once to expand enough cells for further experiments. The primary cells from these samples were used in both single-cell sorting and $CD44^+CD24^{-/dim}$-based cell sorting.

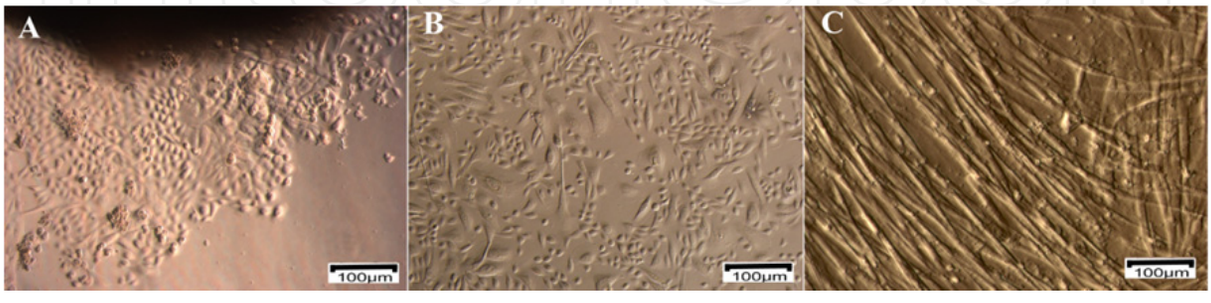

Figure 1. Primary breast cells derived from malignant tumors. (A) Primary cells began to migrate from tumors. (B) Primary cells rapidly proliferated with two main shapes indicating stromal and epithelial-like cells. (C) Cell populations were subcultured for a second passage and cultured in DMEM/F12 supplemented with 10% FBS.

3.2. Single cell-based culture, cell selection and EMT

Primary cells were individually sorted into the wells of 96-well plates. A total of 2880 wells were used for one primary cell population. Single cells were cultured in M171 medium containing MEGS for 2 weeks. There were 14.67±5.13 colonies formed per 96-well plate. Single cells in other wells did not proliferate or died (n=3). Similarly to primary culture, there were two kinds of cell clones. One kind of cell clone was epithelial-like, and the other was mesenchymal-like. To enrich mesenchymal-like cells and eliminate epithelial-like cells, we changed the medium from M171 medium containing MEGS to DMEM/F12 supplemented with FBS that was suitable for mesenchymal-like cells. After all epithelial-like cell clones died in 24 h, mesenchymal-like cell clones continued to expand to 70–80% confluence and were then subcultured. From one sample, 6.33±3.06 mesenchymal-like cell clones were derived per 96-well plate (Fig. 1C). These cell clones were subcultured continuously for three to five passages to identify the cell clone with the earliest EMT. At the third passage, EMT began to occur in some cell clones. These early EMT cell clones were considered as BCSCs. EMT occurred randomly in wells, in which some cells changed shape from mesenchymal (Fig. 2A) to epithelial (Fig 2. B). This process continued until all cells showed epithelial-like shapes (Fig. 2C). We randomly selected one cell clone to analyze the characteristics of BCSCs. These procedures were performed similarly for all three samples.

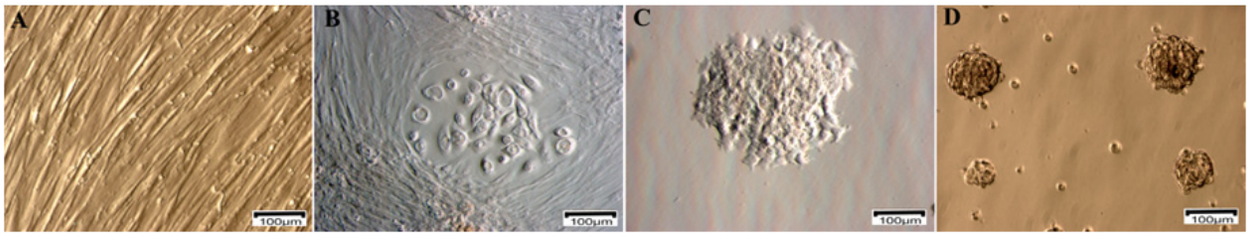

Figure 2. EMT of primary cells. Nearly 100% of cells exhibited a mesenchymal-like shape (A) when cultured in DMEM/F12 supplemented with 10% FBS. EMT of some cells (B) formed an islet of epithelial-like cells. Transitioned cells rapidly proliferated as condensed colonies (C).

3.3. Immunophenotype, rhodamine 123 efflux and mammosphere formation

We randomly selected three cell clones from the three samples to analyze the immunophenotype, rhodamine 123 efflux and mammosphere formation. The results are shown in Fig. 3. BCSC candidates showed a highly homogenous $CD44^{+}CD24^{-}$ phenotype (Fig. 3A) with more than 98% positive (98.82±0.72%) (n=3). These cell clones also contained a subpopulation (SP- Rhodamine 123 efflux phenotype) that showed more than 66.64±8.51% (n=3) (Fig. 3B). In addition, cell clones could form mammospheres when cultured in serum-free medium (Fig. 3C). Compared with BCSCs from $CD44^{+}CD24^{-/dim}$ cell sorting, the cell population was only 90.10±4.12% $CD24^{-}$ and 8.19±3.38% $CD24^{dim}$ (Fig. 3D). Notably, $CD44^{+}CD24^{-/dim}$-sorted BCSCs contained a smaller SP than single-cell sorted BCSCs (34.56±3.48% vs. 66.64±8.51%) (Fig. 3E). However, the two kinds of BCSCs strongly exhibited mammosphere formation (Fig. 3F).

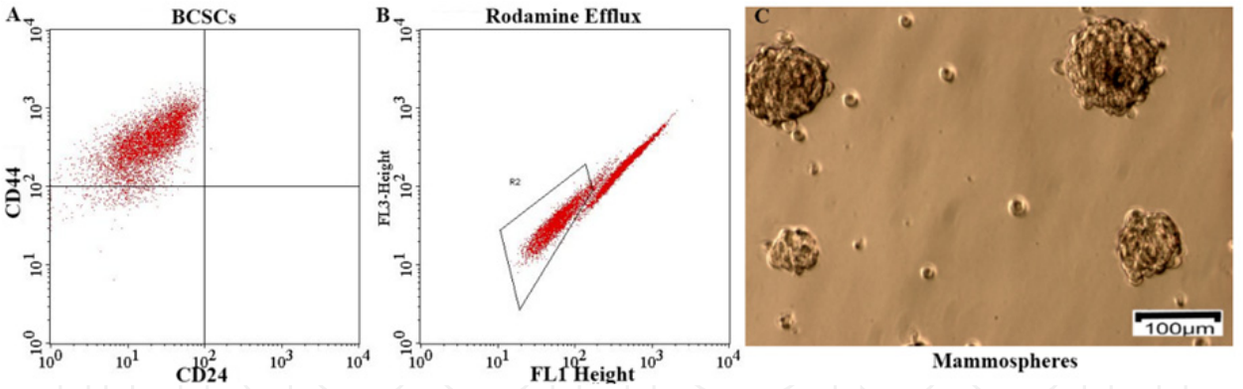

Figure 3. Flow cytometric analyses of immunophenotype and rhodamine 123 efflux. Cell clones exhibited the characteristics of BCSCs with near 100% $CD44^{+}CD24^{-}$ (A), and were more than 50% SP-positive (D). Marker expression and the SP decreased in $CD44^{+}CD24^{-/dim}$-sorted cells. (D; E). However, both types of sorted cells could form mammospheres in serum-free medium (C, F).

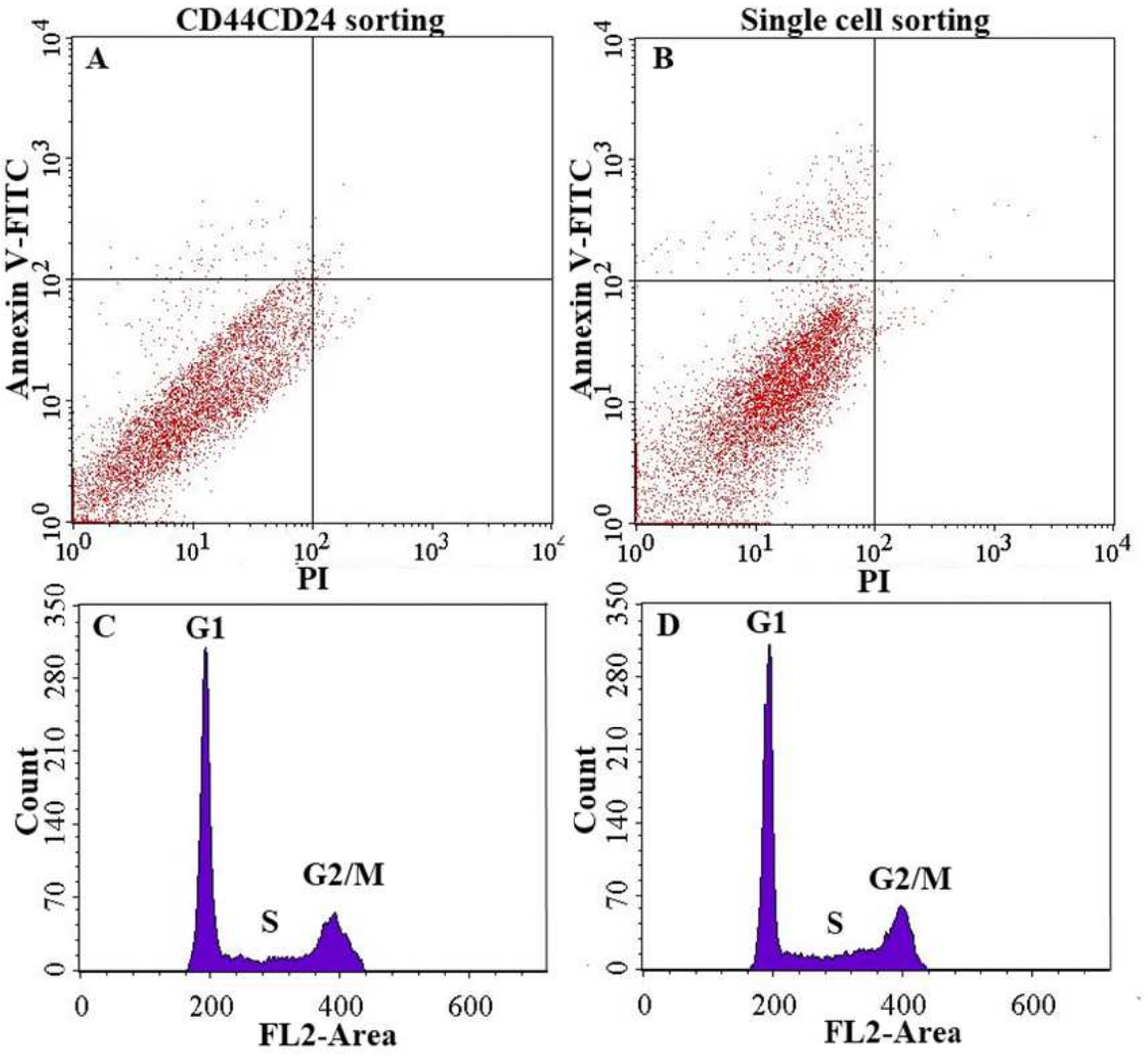

Figure 4. Doxorubicin resistance and the cell cycle of BCSCs isolated by $CD44^{+}CD24^{-}$-sorting and single-cell sorting. At 3 μg/ml doxorubicin, BCSCs obtained from single-cell sorting did not undergo apoptosis (A), whereas apoptosis was observed among $CD44^{+}CD24^{-}$ BCSCs (B). However, the cell cycles of these two populations were not significantly different (C–D).

3.4. Doxorubicin resistance and the cell cycle

BCSCs sorted by two strategies were evaluated in this study. BCSCs from single-cell sorting could resist doxorubicin more than $CD44^{+}CD24^{-/dim}$-sorted BCSCs. At 0 and 1 μg/ml doxorubicin, cells did not undergo apoptosis. However, at 3 μg/ml doxorubicin, no BCSCs underwent apoptosis from single-cell sorting, but there were 2.54±1.29% apoptotic $CD44^{+}CD24^{-/dim}$ BCSCs (Fig. 4A, B). Although these results showed that there was no significant different between BCSCs obtained from single-cell sorting and $CD44^{+}CD24^{-/dim}$-sorting (Fig. 4C, D).

3.5. *In vivo* tumor formation

Tumorigenicity is an essential characteristic of cancer stem cells. In almost all studies, tumor formation at a low number of injected cells is considered as the gold standard for cancer stem cell confirmation. In this study, we injected 1×10^3, 10^4 and 10^5 cells into the mammary pad of NOD/SCID mice. At 1×10^3 cells per mouse, BCSCs were able to form tumors (Fig. 5A, B). To

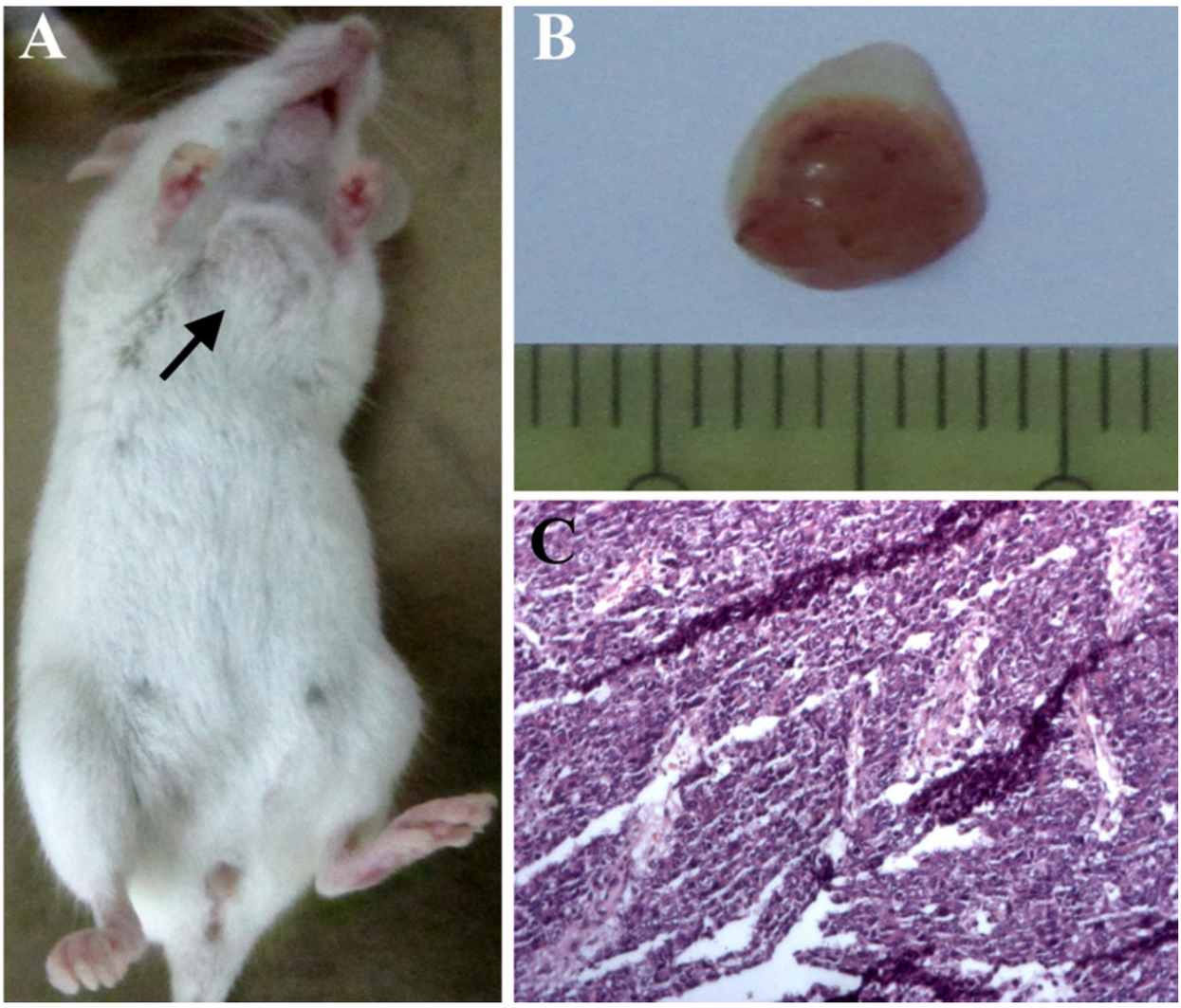

Figure 5. Tumor formation in NOD/SCID mice after injection of 1×10^3 BCSCs. Tumors formed subcutaneously (A) at sizes from 5×6 mm (B), which were analyzed histochemically by HE staining (C).

confirm the histopathology of tumors, 10 μm tumor sections were stained with hematoxylin-eosin (HE). As shown in Figure 5C, tumors exhibited cancer cells with large nuclei. This result was similar to that of BCSCs from $CD44^{+}CD24^{-/dim}$-sorting in our previous studies [41]; [42]; [43].

4. Discussion

BCSCs are the origin of breast tumors. Thus far, the aim of many studies has been BCSC targeting. Recently, preclinical trials have demonstrated that agents targeting BCSCs are more effective than those targeting tumor cells. In all BCSC-based therapies, BCSCs are important for tumor targeting. However, the procedures used to isolate BCSCs are complicated and time consuming. Moreover, all present protocols obtain heterogenous populations of BCSCs. Indeed, sorting CD44+CD24- cells, a population considered as BCSCs, can express various levels of CD44 and CD24. SPs are also considered as BCSCs, but only a subpopulation show a CD44+CD24- phenotype. Thus, the aim of this study was to establish a new protocol to isolate homogenous BCSCs.

Similar to other techniques, our technique also cultured primary cells from malignant tumors as a first step. We successfully cultured 3/5 samples under this condition. Cells rapidly expanded around tumor fragments after 2 weeks. Various cell types appeared in primary culture, including mainly epithelial-like and mesenchymal-like cells. These results were consistent with our previous studies [41]; [42]. The primary cell population may contain at least six cell types including fibroblasts, mammary epithelial cells, mammary epithelial stem cells, breast cancer stem cells, breast cancer cells and stromal cells. To isolate homogenous BCSCs at a high purity, we applied single-cell sorting to individually isolate single cells in the wells of 96-well plates. For each sample, we sorted single cells into 2880 wells in 30 plates. From such a plate, we derived 14.67±5.13 cell clones, while other cells could not proliferate or died. Using this method, we can select cells that rapidly proliferate and survive when cultured alone. Indeed, normal cells and differentiated cells hardly proliferate when seeded as single cells. We considered that BCSCs existed among these clones. By changing the medium from M171 medium containing MEGS to DMEM/F12 supplemented with 10% FBS to select mesenchymal-like cell clones, all cell clones with an epithelial phenotype died and cell clones with a mesenchymal phenotype survived and proliferated.

There were 6.33±3.06 surviving cell clones per plate, with a mesenchymal-like shape. They were continuously subcultured for three to five passages, and cell clones that underwent the earliest EMT were chosen for further study. The results showed that cell clones will the earliest EMT occurred at the third passage. EMT resulted in cells spreading out to form an area with epithelial-like cells among mesenchymal-like cells. After 72 h, all cells transitioned into epithelial cells. We randomly selected three cell clones from a sample to analyze the characteristics of BCSCs. All three cell clones after EMT showed the properties of BCSCs. The cell population exhibited the common CD44+CD24- phenotype of BCSCs at 98.82±0.72%. This population was used to evaluate the SP by rhodamine 123 efflux. The

results showed that the SP was CD44+CD24-/dim. To assess the multidrug-resistance property, we checked and compared with two other techniques, we recognized that BCSCs from single-cell sorting with higher antitumor drug resistance but the same tumor formation in NOD/SCID mice compared with CD44+CD24-/dim-sorting.

Single-cell sorting combined with subculture to isolate EMT cell clones exhibited several benefits for selection of BCSCs. Indeed, single-cell cloning is considered as the best technique for selection of a homogenous cell population. This technique is popular for cell cloning of hybridomas for monoclonal antibody production. However, in monoclonal antibody production, almost all studies use limited dilution or ring/syringe isolation. These two techniques have some limitations; particularly the low efficiency in dilution to obtain single cells in each well and it is laborious, time-consuming and uneconomical to screen samples with a low concentration of desired cells. Using the index sorting of the FACSJazz, it is easy to seed one cell in each well of 96-well plates. Single-cell sorting offers a new tool to efficiently and rapidly perform cell cloning.

In the next step, we cultured single cells to obtain cell clones for subculture. In mammalian cell culture, single-cell culture is usually suitable for transformed cells and immortal cell lines. Normal cells usually undergo apoptosis after 50±10 divisions because of the Hayflick limitation [27]. Thus, single-cell culture is only suitable for immortal cells. Indeed, in our study, when primary cells from tumors were individually cultured, some types of non-immortal cells can be eliminated after culture and subculture. Using single cell-based culture and sub-culture, almost all cell clones of stromal, epithelial and breast cancer cells can be lost over time. Thus, after three passages, some cell clones survive and can form continuous cultures. There were two cell types, namely epithelial-like cells and mesenchymal-like cells. We considered that mammary epithelial stem/progenitor cells exhibited the epithelial shape and breast cancer stem cells exhibited the mesenchymal shape. Mammary epithelial stem cells cannot survive in medium without hydrocortisone and EGF, whereas BCSCs do not depend on hydrocortisone or EGF [18]. Moreover, serum can inhibit the growth of normal epithelial cells from mammary tumors [17]. To eliminate mammary epithelial stem/progenitor cells, we changed the culture medium to DMEM/F12 supplemented with 10% FBS. After 48 h culture in this new medium, all epithelial-like cell clones did not survive, while all mesenchymal-like cell clones survived.

We propose that at this step, we successfully selected BCSC clones or BCSC-like clones. In the next experiment, we selected the strongest BCSC clone. The strongest BCSC clone was selected based on the time of EMT. EMT is related to the initiation of metastasis and cancer by BCSCs [2]; [52]; [20]; [53]; [48]; [45]; [35]. EMT can result in cells with stem cell properties [33]. Notably, Morel et al. (2008) could obtain BCSCs from EMT [37]. In a recent study, Blick et al. (2010) determined that EMT occurs together with the CD44+CD24-/dim phenotype of BCSCs [6]. Thus, in this study, cell clones that rapidly transitioned from mesenchymal to epithelial phenotypes were chosen as the most appropriate BCSC clone.

However, based on immunophenotypic and rhodamine 123 analyses, we found that BCSCs from single-cell sorting might not be homogenous. Indeed, a few cells did not exhibit the

CD44+CD24- immunophenotype or SP phenotype, indicating that BCSCs had differentiated into other cell types during proliferation. Such differentiation may be induced by medium containing serum. Some studies show that serum can induction differentiation of BCSCs [47]; [23]. Moreover, single cell-based culture easily induces differentiation [36]. Thus, single-cell sorting can obtain a pure population of BCSCs that must be maintained in a suitable medium to inhibit spontaneous differentiation.

5. Conclusion

Single-cell sorting is suitable for isolation of BCSCs to obtain a homogenous population for further experimentation and BCSC-targeting therapies. BCSCs obtained by this technique exhibit high purity, high resistance to doxorubicin, and form tumors in NOD/SCID mice at a low cell number. Compared with CD44+CD24- sorting, mammosphere culture and SP-based sorting, single-cell sorting in combination with subculture enables selection of EMT cell clones that give rise to a BCSC population with advantages such a homogenous population, higher doxorubicin resistance and mammosphere formation at high levels. However, spontaneous differentiation in culture is a problem that needs to be addressed. Single-cell sorting offers a new technique to detect and isolate BCSCs as well as other cancer stem cell types.

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